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An Anomalous Distance Dependence of Intra-Protein Chlorophyll-Carotenoid Triplet Energy Transfer

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ABSTRACT In the light harvesting chlorophyll pigment-proteins of photosynthesis, a carotenoid is typically positioned within a distance of ~4 Å of individual chlorophylls or antenna arrays, allowing rapid triplet energy transfer from chlorophyll to the carotenoid. This triplet energy transfer prevents the formation of toxic singlet oxygen. In the cytochrome b_6f complex of oxygenic photosynthesis that contains a single chlorophyll *a* molecule, this chlorophyll is distant (14 Å) from the single β-carotene, as defined by X-ray structures from both a cyanobacterium and a green alga (1, 2). In spite of this separation, rapid (< 8 ns) long-range triplet energy transfer from the chlorophyll *a* to β-carotene is documented in the present study, in seeming violation of the existing theory for the distance dependence of such transfer. We infer that a third molecule, possibly oxygen trapped in an intra-protein channel connecting the chlorophyll *a* and β-carotene, can serve as a mediator in chlorophyll-carotenoid triplet energy transfer in the b_6f complex.

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The membrane-bound cytochrome b_6f complex in oxygenic photosynthesis (Fig. 1A) mediates electron transfer between the reaction centers of photosystems I and II and facilitates coupled proton translocation across the membrane. It contains a *single* chlorophyll (Chl) *a* molecule that is known to produce highly toxic singlet oxygen ($^1O_2$) as the result of energy transfer from its excited triplet state to the oxygen molecule (5). The recent x-ray structures of the b_6f complex show that the β-carotene is 14 Å from the Chl *a* (1, 2)—too far for protection against singlet oxygen formation via the conventional mechanism of direct quenching of the Chl *a* triplet excited state by β-carotene (6). We have recently reported that, unlike other known Chl-containing protein complexes, the formation of singlet oxygen by the Chl *a* in the b_6f complex is reduced by a factor of ~25 by the unusually short singlet excited state lifetime of the Chl *a* (7). The time-resolved optical experiments reported in the current work reveal that additional protection in the cytochrome b_6f complex is provided by rapid triplet-triplet excitation energy flow between the Chl *a* and β-carotene that unexpectedly occurs over the large

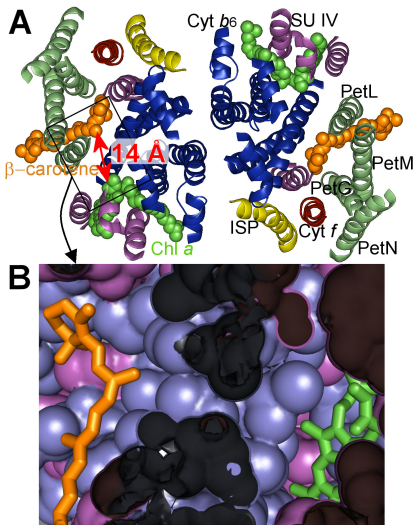

FIGURE 1 ***(A)*** **The lumenal side view of the dimeric cytochrome b_6f complex (1).** ***(B)*** **A cross-section (black) cut through the structure reveals a cavity formed by hydrophobic residues that may serve as an oxygen channel for shuttling triplet excitation energy from the Chl *a* (green) to β-carotene (orange). Five hydrophobic residues (Ile, Leu, Phe, Met and Val) are proposed to form an oxygen channel (blue) (3, 4).**

distance, and must involve an unconventional mechanism.

To examine triplet-triplet energy transfer in the cytochrome b_6f complex, the dynamics of absorption changes associated with the excited states of the Chl *a* and β-carotene were probed. Samples of conventionally purified cytochrome b_6f complex, as well as of complex that was further refined to an ultra-pure state by dissolving diffraction-quality crystals, were excited within the Chl *a* absorption band at 660 nm and probed at 520 nm and 670 nm under aerobic conditions (Fig. 2). The 520 nm kinetic profile in both samples features a major exponential component with a decay time of 2.6±0.5 μs (Figs. 2A, B). The decay-associated spectrum (DAS) of the 2.6 μs component formed a distinct band centered at 520 nm (Fig. 2C). The lifetime and spectral shape of this component are consistent with the triplet-singlet (T-S) spectrum of a carotenoid molecule, which has been shown to have a lifetime of 2-4 μs under aerobic conditions (8). The excitation spectrum of the 2.6 μs component mimicked the Q_y absorption band of the Chl *a* centered at 670 nm, indicating that the triplet excited state of carotenoid ($^3Car^*$) is created as the result of the Chl *a* excitation. No signal associated with $^3Chl^*$ was resolved in the complex purified through crystallization (Fig. 2A, 670 nm profile). It is inferred that the characteristic time for triplet energy transfer from the Chl *a* that is integral to the cytochrome b_6f complex to the β-carotene is shorter than the time resolution (~ 8 ns) of the instrument, so that the signal escapes detection. For conventionally purified complex that was not further purified by crystallization, and which thereby contains a small amount (~20 % of the total Chl *a*) of adventitiously bound Chl, the photo-bleached signal probed at 670 nm decays with a lifetime of ~110 ns (Fig. 2D). This is consistent with the expected lifetime of the triplet excited state of a monomeric Chl ($^3Chl^*$) under aerobic conditions where the chlorophyll triplet energy is transferred to the triplet-singlet transition of molecular oxygen (5) and indicates that this signal originates entirely from Chl non-specifically bound to the b_6f complex.

These experimental results unambiguously demonstrate that a substantial amount of $^3Car^*$ is formed from excitation of the Chl *a* in the cytochrome b_6f complex, and imply that an effective triplet-triplet energy transfer channel exists between

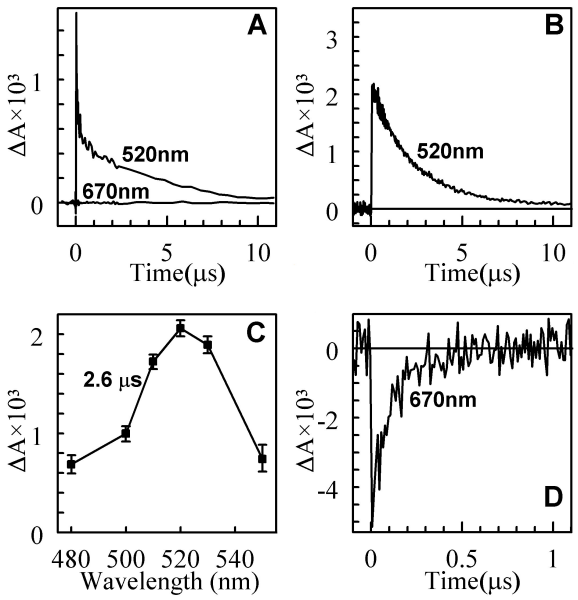

FIGURE 2 **(*A*) Time-resolved transient absorption difference profiles probed at 520 nm and 670 nm, which represent triplet $^3Car^*$ and $^3Chl^*$ population dynamics. The b_6f complex purified via crystallization was excited at 660 nm under aerobic conditions. The amplitude of the absorbance changes at 670 nm is zero relative to the noise level. (*B*) Transient absorption measured at 520 nm for the conventionally purified complex under the same conditions. (*C*) The amplitude of the 2.6 μs component associated with $^3Car^*$ formation shown in panel *B* as a function of probe wavelength. (*D*) Transient absorption signal probed at 670 nm for the conventionally purified complex is not zero and stems from non-specifically bound chlorophylls.**

the Chl *a* and β-carotene. No signal associated with the formation of the integral $^3Chl^*$, nor rise time in $^3Car^*$ population (Fig. 2A, B), was resolved in these experiments, which sets the upper limit for this triplet-triplet energy transfer to < 8 ns (the time resolution of our experimental setup.

Using the theory of Dexter (9), we estimated that the rate of the direct triplet-triplet energy transfer from the $^3Chl^*$ to β-carotene in the cytochrome b_6f complex should be ~$(0.3\ ms)^{-1}$, which is ~5 orders of magnitude slower than the upper limit of the $^3Car^*$ formation time observed in the b_6f complex. Thus, not surprisingly, the conventional mechanism of singlet oxygen protection by direct triplet-triplet energy transfer process between the Chl and Car separated by 14 Å does not function in the cytochrome b_6f complex.

It is proposed that oxygen mediates triplet energy transfer between the Chl *a* and β-carotene. Oxygen can effectively accept triplet excited state energy from $^3Chl^*$, forming singlet oxygen (5), and it is well known that singlet oxygen in solvents can be effectively quenched by a carotenoid, promoting the latter into the triplet excited state (6, 10).

To facilitate rapid energy transfer, oxygen could be confined in its diffusive motion to an intra-protein channel connecting the Chl *a* and Car, causing a significant increase in the local oxygen concentration and the rate of the oxygen-mediated Chl *a* triplet state quenching. An oxygen channel has been described that facilitates oxygen transfer within cytochrome *c* oxidase, which catalyzes the reduction of oxygen to water (3). Simulations of this process by molecular dynamics (4) show that molecular oxygen shuttles along a single well-defined ~15 Å long pathway in a time on the order of tens of picoseconds, with a low probability of escape from this channel. The involvement of mobile oxygen in the triplet-triplet energy transfer in the cytochrome b_6f complex isolated from a cyanobacterium would be consistent with the absence of the $^3Car^*$ signal at 77 K reported by Peterman et al. (11)—the mobility of oxygen would be greatly impeded at low temperatures. We have confirmed this result (data not shown).

Both experimental studies and molecular dynamic simulations imply that an effective intra-protein oxygen channel could be formed by hydrophobic residues (3, 4). Structural analysis of the cytochrome b_6f complex reveals that there is, indeed, an open pathway surrounded primarily by hydrophobic residues (Fig. 1B). Since the rate of the $^1O_2$ quenching by β-carotene is more than 2 orders of magnitude greater than the reactivity of $^1O_2$ toward the surrounding amino acids (12), this mechanism for triplet energy transfer to β-carotene would allow it to serve a protective function. The reason for distant placement of the necessary protective β-carotene relative to the chlorophyll *a* remains a question.

METHODS

Purification and crystallization of the cytochrome b_6f complex from *Mastigocladus laminosus* is described in detail elsewhere (13). Conventionally purified complexes (i. e. purified without crystallization) contained approximately ~1.2 Chl *a* molecules per cytochrome *f*. Control experiments were performed on x-ray diffraction quality single crystals of the cytochrome b_6f complex dissolved in a buffer. These samples had a stoichiometry of Chl *a* 1.0:1 relative to cytochrome *f*. All complexes were in a functionally active form.

Transient absorption difference measurements were carried out by laser flash photolysis using alternatively ~20 ns or 100 fs fwhm excitation pulses at ~660 n. Time resolution was limited only by the light detectors (~8 ns).

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